REMARKS

Claims 11, 12 and 16 are pending in the instant application. Claims 11, 12 and 16 have been rejected. Reconsideration is respectfully requested in light of the following remarks.

The rejection of claims 16, 11 and 12 under 35 U.S.C. § 103(a) as being obvious over the teachings of Yamashita et al. has been maintained.

Applicants respectfully traverse this rejection.

At the outset, in response to the Examiner's invitation to submit objective evidence demonstrating that a subset of all carcinomas would **not** overexpress PLA2, Applicants are providing herewith a publication by Funkakoshi et al. (Pancreas 1991 6(5):588-594). This publication teaches that serum PLA2 levels were increased significantly in patients with acute pancreatitis and that the levels correlated with disease severity in patients with pancreatic cancer. However, they teach that serum PLA2 levels were within normal range in patients with other malignant tumors, diabetes mellitus and chronic liver disease. See Abstract. Further, at page 590, they state that in patients with pancreatic cancer, "serum PLA2 concentrations varied with the severity of the disease." This is contrasted with serum PLA2 concentrations in

patients with other malignant tumors which were taught to be within the normal range or only slightly increased. Also see Abstract and Figure 1. Evidence disclosed in this reference supported the authors conclusion that measurement of serum PLA2 is clinically useful for diagnosing and monitoring pancreatitis. No correlation between PLA2 levels and diagnosing other malignant tumors was suggested.

Thus, this reference provides objective evidence that serum PLA2 concentrations are **not** nor would be expected by the skilled artisan to be elevated in a subset of all carcinomas. Further, this reference provides objective evidence that overexpression of PLA2 can **not** be used to monitor progression of all carcinomas. This reference also clearly shows the teachings of Yamashita et al. are not representative of the "preponderance of the evidence" and that the "preponderance of the evidence" is not demonstrative of the association of carcinomas in general with PLA2 overexpression.

MPEP § 2142 is quite clear; the decision of patentability must be based upon consideration of all the evidence, including that submitted by the Examiner as well as the Applicant. Evidence of record herein, when considered as a whole, clearly shows that there is no predictability from the prior art with respect to PLA2

overexpression being useful in monitoring progression of ovarian or testicular cancer.

Applicants also respectfully disagree with the Examiner's suggestion at page 2 of the Office Action that the claims are not drawn to monitoring at selected times. Claim 16 of the instant application is drawn to a method of **monitoring** progression of ovarian or testicular cancer in a patient by measuring PLA_2 levels in biological samples obtained from the patient **at selected times;** and then comparing these measured PLA2 levels to determine whether these has been an increase in the measured levels of PLA_2 in the patient over time which is indicative of progressive ovarian or testicular cancer, a decrease in the measured levels of PLA_2 in the patient over time which is indicative of remission or response to therapy of the ovarian or testicular cancer or no change in the measured levels of PLA_2 in the patient over time which is indicative of stabilization of the ovarian or testicular cancer. Accordingly, contrary to the Examiner's suggestion, arguments relating to these limitations as distinguishing the present invention from the cited prior art teaching of Yamashita et al. are relevant to the invention as claimed.

Thus, since the prior art fails to provide any reasonable

expectation of success with respect to the instant claimed invention and fails to teach or suggest all the limitations of the claimed invention, no *prima facie* case of obviousness has been established. Withdrawal of this rejection under 35 U.S.C. § 103(a) is therefore respectfully requested in light of the objective evidence provided by Applicants herewith and the above remarks.

Conclusion

Applicants believe that the foregoing comprises a full and complete response to the Office Action of record. Accordingly, favorable reconsideration and subsequent allowance of the pending claims is earnestly solicited.

Respectfully submitted,

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Clinical Usefulness of Serum Phospholipase A_2 Determination in Patients with Pancreatic Diseases

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Summary: A new kit for radioimmunoassay of serum phospholipase A_2 (PLA_2) with monoclonal antibody (S-0932, Shionogi, Osaka, Japan) was used to examine PLA_2 levels in patients with various diseases. Patients with acute pancreatitis showed significantly increased serum PLA_2 levels. In patients with chronic pancreatitis, significant correlations were observed between the levels of factors evaluated by the secretin test and serum PLA_2 levels. In patients with pancreatic cancer, serum PLA_2 levels varied with disease severity. Serum PLA_2 concentrations were within the normal range in patients with other malignant tumors, diabetes mellitus, and chronic liver diseases but were increased in patients with chronic renal failure. S-Sepharose column analysis of sera showed a small peak of pro-PLA_2 and a large peak of PLA_2 in sera from patients with severe acute pancreatitis, but a large peak of pro-PLA_2 in healthy controls and patients with other diseases. On G-100 gel filtration, high-molecular-weight PLA_2 immunoreactivity was detected in sera of patients with chronic renal failure, whereas a single peak of PLA_2 immunoreactivity coinciding with that of standard PLA_2 was detected in sera of patients with acute pancreatitis. These results suggest that (a) measurement of serum PLA_2 is clinically useful for diagnosis and monitoring of pancreatitis, (b) active PLA_2 in the circulation is dominant in severe acute pancreatitis, and (c) the kidney may be the main site of PLA_2 degradation or excretion. Key Words: Phospholipase A_2—Pancreatic diseases.

Pancreatic phospholipase A_2 (PLA_2; EC 3.1.1.4) is secreted into the pancreatic juice by pancreatic acinar cells as a proenzyme (pro-PLA_2), which is activated by trypsin, and acts as a digestive enzyme. It is also known to be involved in development of necrotizing pancreatitis (1,2). Numerous reports have indicated the importance of PLA_2 in development and aggravation of acute pancreatitis (1–6). PLA_2 from an injured pancreas is believed to accumulate the serum at high concentration and to hydrolyze the phospholipids in cell membranes (1–6). In addition, the free fatty acid liberated from phospholipid may be converted through intermediates to potent pharmacologic mediators such as prostaglandins (7–9), and thromboxane (10). The enzyme may also be involved in development of shock and of pulmonary and cerebrospinal lesions (11–14).

Several reports have described PLA_2 activities in human sera (4–6,11,15–17), but little is known about the enzyme contents of human sera (18,19). Until recently, serum PLA_2 has been difficult to measure because no specific substrate is available for its en-

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zymatic determination. In the present study, we used a new radioimmunoassay (RIA) kit with monoclonal antibody (MoAb) to PLA_2 (S-0932, Shionogi, Osaka, Japan) for measuring serum PLA_2 (20) to evaluate the usefulness of serum PLA_2 determination in patients with pancreatic diseases. We also examined the heterogeneity of PLA_2 in serum by gel filtration and ion-exchange chromatography.

MATERIALS AND METHODS

Patients

Thirteen of the patients had acute pancreatitis, 77 had chronic pancreatitis (2 after total pancreatectomy), and 53 had pancreatic cancer. The severity of acute pancreatitis was clinically staged according to Ranson's prognostic criteria (21). The diagnosis of chronic pancreatitis was based on radiologic findings of pancreatic calcification, irregular dilatation of the pancreatic duct detected by endoscopic retrograde pancreatography, and hypofunction of the exocrine pancreas determined by the secretin test (22). The secretin test was performed using a Dreiling double-lumen tube. One hundred units of Secrepan (Eisai, Tokyo, Japan) was injected as secretin by drip infusion for 60 min, and the duodenal juice was collected every 10 min during the last 30 min. Patients were divided into two groups (group I confirmed to have chronic pancreatitis, and group II suspected of having chronic pancreatitis) according to the criteria of the Japanese Society of Gastroenterology (23). Pancreatic cancer was proved histologically. Other groups studied included 32 patients with diabetes mellitus, 55 patients with chronic liver diseases, 45 patients with hepatocellular carcinoma, 19 patients with chronic renal failure, 51 patients with other malignant neoplasms, and 74 healthy subjects. All blood samples were collected during the fasting state.

RIA of PLA_2

RIA of PLA_2 was performed as described previously (20) with a PLA_2-RIA kit. MoAb (mouse ascites no. 1008) for PLA_2, specific for pancreatic PLA_2, cross-reacted 49% with pro-phospholipase A_2 (pro-PLA_2) but did not cross-react with other pancreatic enzymes, such as elastase 1, trypsin, chymotrypsin, lipase, and amylase. The sensitivity of the assay was 100 ng/dl. The intra- and interassay coefficients of variation were 1.6–4.8 and 2.5–5.3%, respectively. The normal range, defined as 2 SD, calculated by Hoffmann method from the mean for healthy subjects (n = 74) is 170–435 ng/dl.

Gel filtration and ion-exchange chromatography

All chromatography procedures were performed at 4°C. Serum was applied to a Sephadex G-100 (superfine) column (1 × 100 cm) equilibrated, and developed with 10 m*M* Tris-HCl buffer (pH 7.5) at a flow rate of 10 ml/h. Fractions of 1.5 ml were collected and their PLA_2 and optical density at 280 nm were measured. The column was calibrated with blue dextran, PLA_2, pro-PLA_2, and $Na^{125}I$.

Serum was also applied to a column (1 × 2 cm) of S-Sepharose (Pharmacia, LKB, Uppsala, Sweden), previously equilibrated with 10 m*M* Tris-HCl buffer pH 7.5, 0.05% CHAPS. The column was washed with 2 ml equilibrating buffer, and material was then eluted with 40 ml of a linear gradient of 0–0.2 *M* NaCl in the equilibrating buffer. Fractions of 1 ml eluate were collected at a flow rate of ~12 ml/h, and their PLA_2 contents were measured. The column was calibrated with pro-PLA_2 and PLA_2. As shown in Table 1, the sera analyzed were obtained from 3 healthy controls, 3 patients with pancreatitis, 1 patient with pancreatic cancer, and 1 patient with chronic renal failure. Recovery of PLA_2 immunoreactivity of each serum specimen from the column was ~92% (86–104%), indicating that the serum levels given by PLA_2-RIA coincided with the sum of

TABLE 1. *Serum levels of PLA_2 immunoreactivity and proportions of pro-PLA_2 and PLA_2 estimated by ion-exchange chromatography in various subjects measured by PLA_2 RIA*

Parameter	Healthy subjects			Pancreatitis			PC	CRF
	1	2	3	1	2	3		
PLA_2 level (ng/dl)	390	310	350	540	950	4,740	8,880	4,320
Pro-PLA_2 (%)	95	92	95	88	47	31	92	98
PLA_2 (%)	5	8	5	12	53	69	8	2

PLA_2, phospholipase A_2; pro-PLA_2, procnzyme-PLA_2.
Pancreatitis: Patient 1, pancreatitis after endoscopic retrograde pancreatography. Patient 2, active phase of chronic pancreatitis; patient 3, acute severe pancreatitis. PC, pancreatic cancer; CRF, chronic renal failure; RIA, radioimmunoassay.

the immunoreactivities eluted from the column. PLA_2 was purified from pancreatic juice as described by Nishijima et al. (24). Pro-PLA_2 was purified from pancreatic juice as described by Grataroli et al. (25) except that the material was chromatographed twice on CM-cellulose.

Analysis of data

Values were expressed as the mean ± SE. Results were analyzed by one-way analysis of variance (ANOVA); $p < 0.05$ was considered significant.

RESULTS

Serum PLA_2 levels in various diseases

Patients with acute pancreatitis had significant elevated levels of serum PLA_2 (Fig. 1). In patients with chronic pancreatitis, the serum PLA_2 concentration was low in the stage of severe exocrine dysfunction in group I but high in the stage of acute exacerbation in both groups I and II. In patients after total pancreatectomy, no serum PLA_2 was detectable. Significant correlations were observed between each of the factors evaluated in the secretin test and serum PLA_2 concentrations [$F(4,63) = 6.65$, $p < 0.01$] (Fig. 2). In patients with pancreatic cancer, the serum PLA_2 concentration varied with the severity of disease. The serum PLA_2 concentrations were within the normal range or slightly increased in patients with other malignant tumors, diabetes mellitus, and chronic liver diseases, but in patients with chronic renal failure the serum PLA_2 concentrations were elevated.

Heterogeneity of serum PLA_2

S-Sepharose column analysis showed a single major peak of PLA_2 immunoreactivity coinciding with that of pro-PLA_2 in sera of healthy controls (Fig. 3) and patients with diseases other than acute pancreatitis (Fig. 4). In contrast, sera of patients with severe (patient 3) or moderate (patient 2) acute pancreatitis contained pro-PLA_2 and high levels of PLA_2 (Fig. 5). In patients with chronic renal failure, mainly pro-PLA_2 was detected by S-Sepharose analysis (Fig. 4) and high molecular weight PLA_2 immunoreactivity was detected by G 100 gel filtration (Fig. 6), although gel filtration analysis showed a single peak of PLA_2 immunoreactivity coinciding with that of standard PLA_2 in patients with acute pancreatitis and pancreatic cancer (Fig. 6). RIA of PLA_2 gave a value for the sum of PLA_2 immunoreactivity and cross-reactivity with pro-PLA_2 in sera. The concentrations of pro-PLA_2 and PLA_2 in the sera and their proportions calculated from the peaks obtained by chromatography are shown in Table 1. In all healthy controls, pro-PLA_2 was the major component; PLA_2 was a very small component.

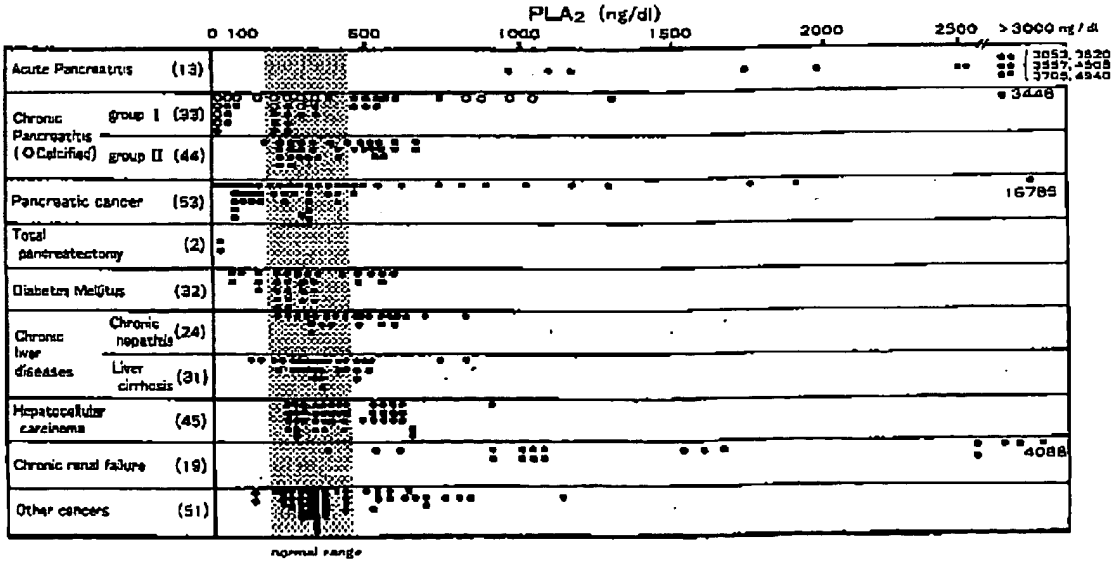

FIG. 1. Serum phospholipase A_2 activities in patients with various diseases. Shaded area: normal range.

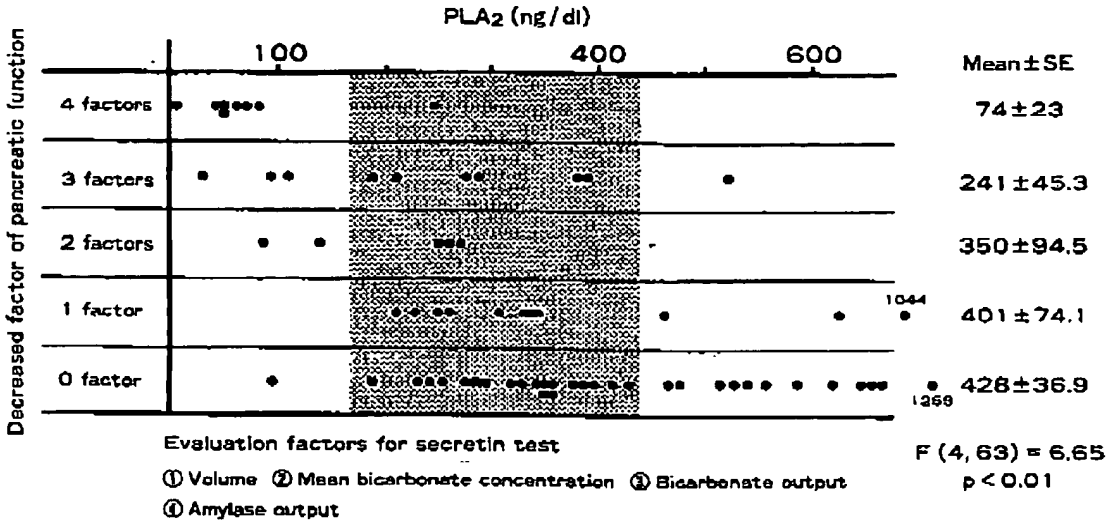

FIG. 2. Relationship between exocrine pancreatic functions and serum phospholipase A_2 activity. Shaded area: normal range of serum PLA_2.

Pro-PLA_2 was also the major component in patients with pancreatic cancer and chronic renal failure. Of the patients with pancreatitis, patient 1 showed proportions of these components (Fig. 4) similar to those in healthy controls (Fig. 5), whereas in patient 2 and more particularly in patient 3, the proportion of PLA_2 was more than that of pro-PLA_2.

DISCUSSION

In the present study, the serum levels of PLA_2 were significantly increased in patients with acute pancreatitis, in patients with chronic relapsing pancreatitis who had abdominal pain, and in patients in the early stage of pancreatic cancer. The increase in serum PLA_2 in patients with pancreatic cancer was considered to be due to obstructive pancreatitis in the early stage. Because the width of the pancreas is ~4–6 cm and the main pancreatic duct runs through the center of the pancreas, even a tumor <2 cm in diameter could obstruct the main pancreatic duct and cause an increase in serum pancreatic enzymes. Therefore, the active stage of pancreatitis and an early stage of pancreatic cancer could be checked by this PLA_2 RIA. In the terminal stage of pancreatic cancer, the serum PLA_2 level decreased be-

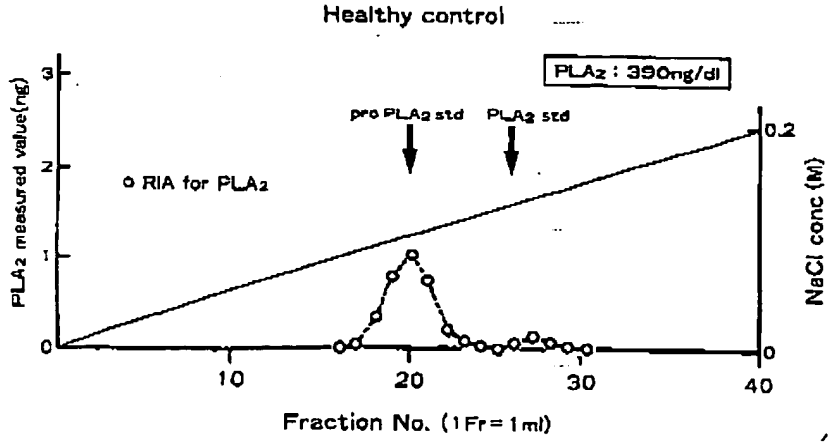

FIG. 3. S-Sepharose chromatography of serum phospholipase A_2 from a healthy subject.

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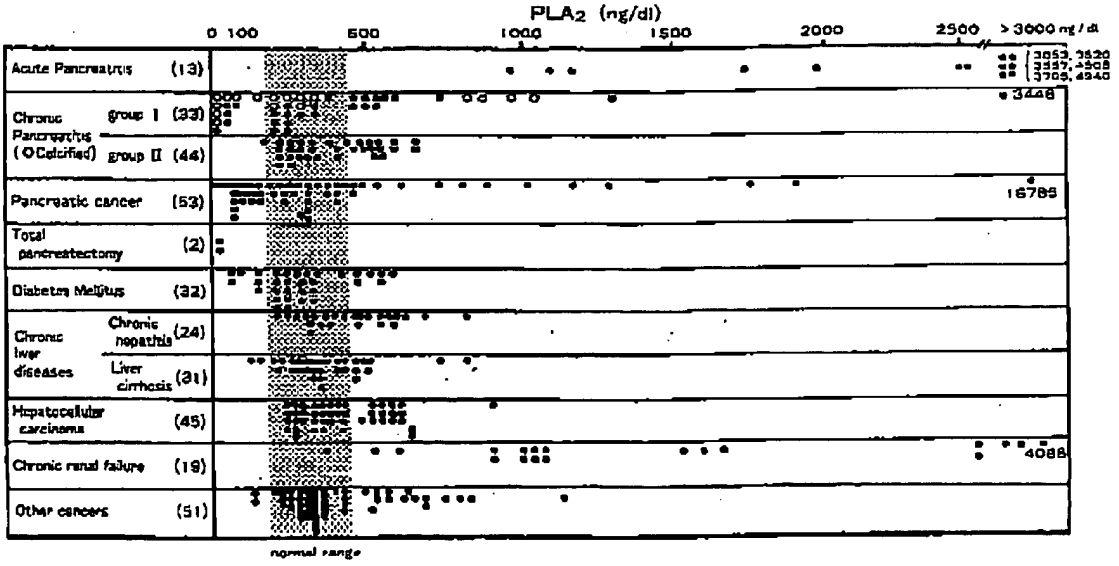

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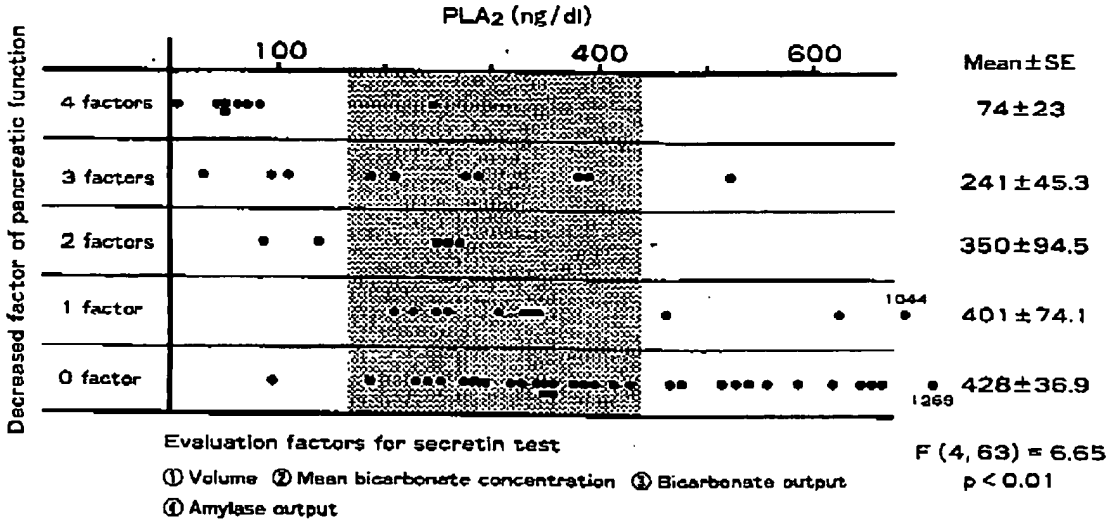

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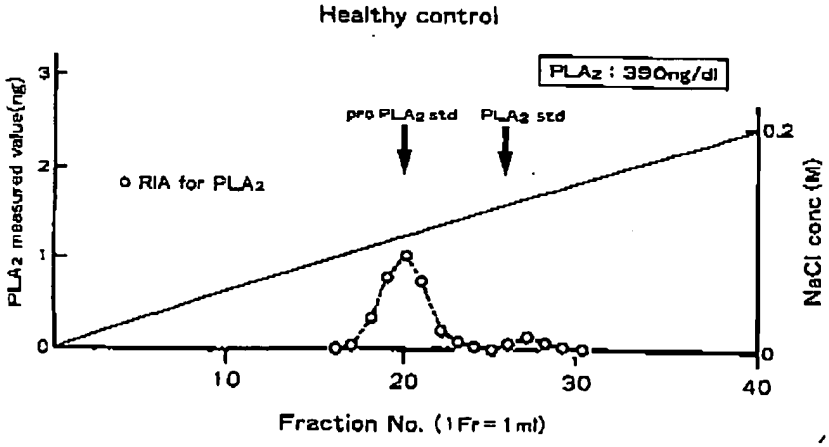

FIG. 3. S-Sepharose chromatography of serum phospholipase A_2 from a healthy subject.

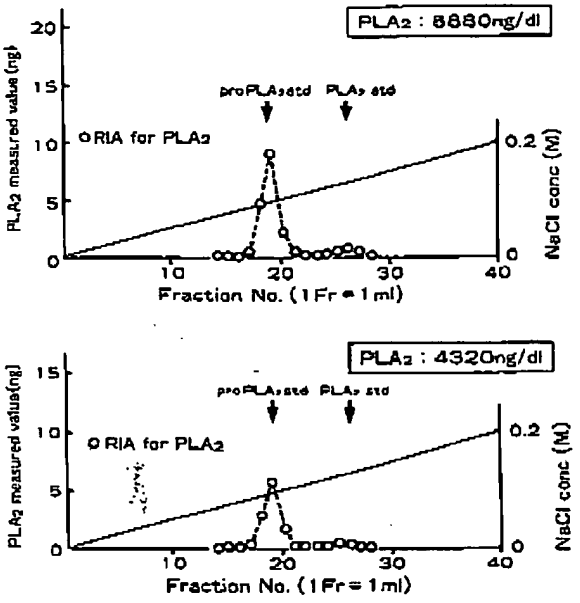

FIG. 4. S-Sepharose chromatography of serum phospholipase A_2 of patients with pancreatic cancer (top) and chronic renal failure (bottom).

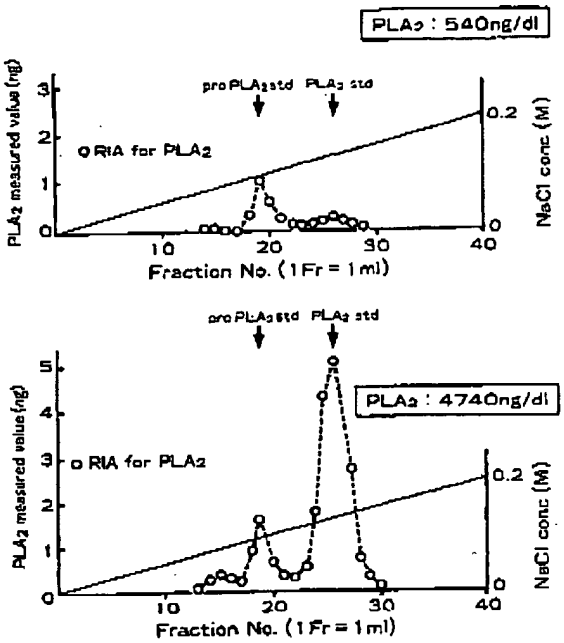

FIG. 5. S-Sepharose chromatography of serum phospholipase A_2 from patients with acute pancreatitis. *Case 1* (top); *case 3* (bottom).

cause of normal pancreatic tissue was replaced by tumor tissue.

Several reports describe PLA_2 catalytic activities in cases of acute pancreatitis (4–6,11,15–17), but little information is available on the significance of enzymatic activity in acute pancreatitis sera except in necrotizing pancreatitis (16–18), chiefly because serum PLA_2 is mainly present as the proenzyme, which is catalytically inactive, or because of the presence of inhibitors of the catalytic activity. Our results by ion-exchange chromatography indicate the presence of the active form of PLA_2 in acute severe pancreatitis. Gel filtration analysis showed that only a single peak of immunoreactivity coincided with standard PLA_2, probably because of the small difference in molecular weight (a difference of only seven amino acids) between pro-PLA_2 and PLA_2 (2), suggesting the absence of inhibitors in the sera. Development of pancreatic necrosis (1,2,26,27) and pulmonary failure (12,13) have been suggested to be the two main causes of acute pancreatitis caused by active PLA_2 release, consistent with a previous hypothesis that active PLA_2 in the sera may exacerbate the general condition during pancreatitis. One of the basic problems in treating patients with acute pancreatitis is to detect those with the severe hemorrhagic form of the disease as early as possible so that adequate treatment can be started immediately. Detection of active PLA_2 in this study suggests the value of measurement of PLA_2 for early assessment of the severity of pancreatitis.

In patients with chronic pancreatitis, the serum levels of PLA_2 paralleled exocrine dysfunction. These results also support the idea that in patients with pancreatic cancer the serum PLA_2 concentration changes in parallel with disease severity. Therefore, we can differentiate exocrine pancreatic function by measuring serum PLA_2.

In patients with chronic renal failure, the serum PLA_2 level was significantly increased, probably due to disturbance of PLA_2 excretion into the urine, because high molecular weight PLA_2, which could represent pro-PLA_2 bound to unknown proteins, was detected by gel filtration analysis.

These results suggest that (a) measurement of serum PLA_2 is useful clinically for diagnosis and monitoring of pancreatitis, (b) active PLA_2 in the circulation is dominant in acute severe pancreatitis, and (c) the kidney may be the main site of PLA_2 degradation or excretion.

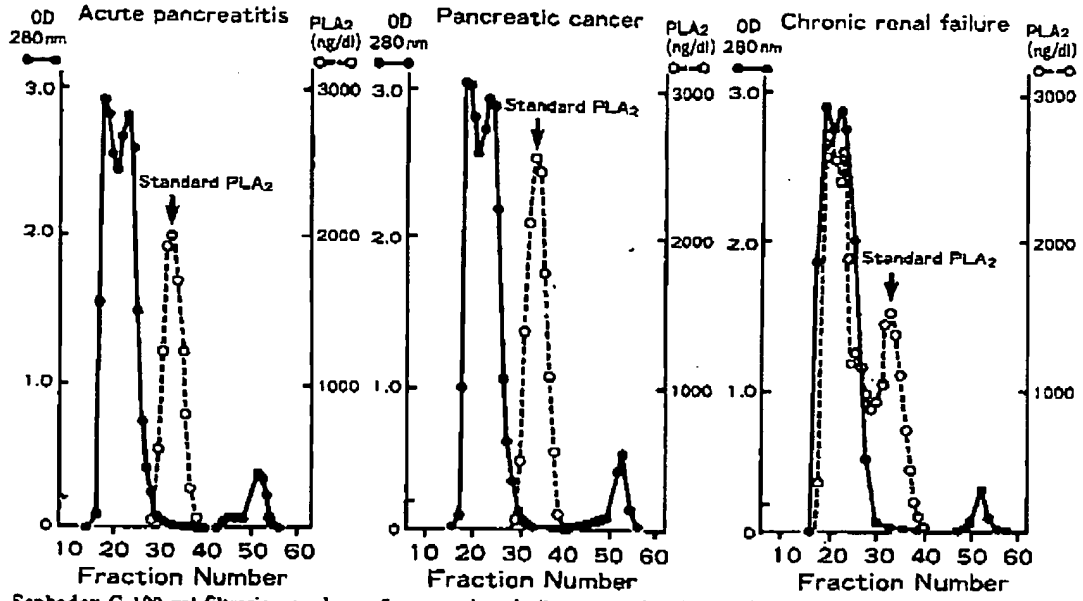

FIG. 6. Sephadex G 100 gel filtration analyse of serum phospholipase A_2 of patients with (left) acute pancreatitis (case 2), (middle) pancreatic cancer, and (right) chronic renal failure.

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